American Journal of Pathology, Vol. 150, No. 6, June 1997

Technical Advance

Lack of Lymphangiogenesis in Human Primary Cutaneous Melanoma

Consequences for the Mechanism of Lymphatic Dissemination

Robert M. W. de Waal,*
Margarethe C. van Altena,* Helmut Erhard,†
Ulrich H. Weidle,‡ Peet T. G. A. Nooijen,* and
Dirk J. Ruiter*

From the Department of Pathology, University Hospital Nijmegen, Nijmegen, The Netherlands; the Department of Dermatology,† University of Würzburg, Würzburg, Germany; and Boehringer Mannheim,‡ Penzberg, Germany*

Cutaneous melanoma has an initial preference for lymphatic spread. Remarkably, melanoma progression toward this metastasizing phenotype is accompanied by intense blood vessel angiogenesis (hemangiogenesis), but lymphangiogenesis, the formation of new lymph vessels in the tumor, has never been reported. To investigate how primary melanoma cells interact with the existing lymphatic microvasculature, and whether lymphangiogenesis occurs, an immunostaining was developed that differentially decorates blood and lymph vessels in frozen tissue sections. The density and distribution of both these vessel types in and around thin (≤1.5 mm) and thick (≥1.5 mm) primary melanoma lesions and in normal and uninvolved skin were determined. Although especially in thick melanoma lesions a significant increase in blood vessel density was observed, lymphatic density remained unaltered, showing that lymphangiogenesis did not occur. Morphological analysis indicated, however, that melanoma progression is accompanied by a sequence of events that involves hemangiogenesis supporting tumor expansion, especially in the vertical growth phase. Often, stromal septa are formed around the blood capillaries in the tumor neovasculature protecting them from invasion. Lymph vessels inside the tumor were infrequently observed. However, subepidermal lymph vessels often seemed to be entrapped and penetrated by the expanding tumor mass. In this way, hemangiogenesis, as the driving force behind tumor expansion, might indirectly increase the chance of lymphatic invasion in the absence of lymphangiogenesis. This model explains the paradox that, although melanoma metastasis seems to require angiogenesis, a consistent relation of prognosis with blood capillary density in primary cutaneous melanoma is lacking. (Am J Pathol 1997, 150:1951–1957)

It has been firmly established that growth of solid tumors is dependent on the development of a tumor vascular bed.[1] Moreover, the transition to an angiogenic phenotype is assumed to be associated with unfavorable prognosis.[2] This notion has led to the concept that the intensity of tumor angiogenesis correlates with metastatic incidence.[3] This was confirmed in a variety of tumors by the finding that a high microvascular density, even in a restricted area of

P. T. G. A. Nooijen was supported by grant NUKC 93-659 from the Dutch Cancer Society.

Accepted for publication February 5, 1997.

Address reprint requests to Dr. Robert M. W. de Waal, Department of Pathology, University Hospital Nijmegen, PO Box 9101, NL-6500 HB Nijmegen, The Netherlands.

the tumor (the so-called hot spot) is a negative prognostic factor.[4]

In other tumor types such as cutaneous melanoma and head-and-neck squamous cell carcinoma, this correlation is less straightforward.[5–7] Although the formation of a neovascular bed in cutaneous melanoma has been regarded as a sign of malignant progression,[8–11] recent extensive analysis has demonstrated that microvascular density is not correlated with metastatic behavior.[6] The reason for this lack of correlation is unclear, but it may be speculated that it is caused by the preference of these tumors to spread initially via the lymphatic route. It is obvious that this preference may be related to a high intrinsic lymphatic density in the tissue where the tumor arose. Little is known about the presence, architecture, and antigenic phenotype of lymph vessels in most tumor types,[12,13] and lymphangiogenesis in tumors has not been reported in the literature.[14] Study of the lymphatic vascular bed has been hampered by the lack of a specific marker for lymphatic endothelium. Most immunohistochemical staining methods that decorate endothelial cells do not discriminate between blood and lymph vessels or do not stain lymphatic vessels at all. We developed a differential immunohistochemical double-staining protocol using antibody reactivity for CD31 (platelet-endothelial cell adhesion molecule-1, PECAM-1) that is present in all types of microvessels,[15,16] in combination with the blood vessel endothelial marker PAL-E.[17] We demonstrate that this staining technique selectively decorates the lymph and the blood vessels in frozen sections of normal skin and primary cutaneous melanoma and that it can be used to determine the densities of both these vascular beds in the same section.

Materials and Methods

Patient Material

Normal skin specimens were obtained at autopsy. Fresh primary melanoma specimens were collected from the operating theater of the Department of Dermatology, University of Würzburg, Germany. Samples of 27 melanomas were analyzed by immunohistochemistry, and 5 melanomas were examined by immunoelectron microscopy (4 superficial spreading melanomas and 1 lentigo maligna melanoma). On hematoxylin-stained sections, tumor thickness, Clark's level of invasion, and the growth phase of each lesion were determined. The depth of invasion according to Clark ranged from level I to level IV, and the vertical tumor thickness according to Breslow varied between *in situ* melanoma and 11.0 mm. Melanomas with Clark level I or II of invasion were considered horizontal growth phase melanomas (HGPMs) when the epidermal component was larger than the dermal one and when the dermal component was arranged in small nests and confined to the papillary dermis. Lesions not fulfilling these criteria and lesions with level III or IV of invasion were considered vertical growth phase melanomas (VGPMs). Patients had not undergone systemic or local treatment. Each sample was split into portions that were either formalin fixed for conventional histopathological diagnosis, snap-frozen for immunohistochemistry, or processed for immunoelectron microscopy.

Antibodies

For immunohistochemistry and immunoelectron microscopy, monoclonal antibodies (MAbs) against CD31 (PECAM-1, 0.5 μg/ml) (British Biotechnology, ITK Diagnostics, Uithoorn, The Netherlands) and CD34 (BioGenex, Klinipath, Duiven, The Netherlands), the endothelial marker MAb PAL-E (undiluted supernatant; Sanbio, Uden, The Netherlands) and polyclonal antisera (pAb) against von Willebrand factor/factor VIII-related antigen (vWF/FVIIIrA; Central Laboratory Blood Transfusion Service, Amsterdam, The Netherlands) and collagen type IV (Coll-IV; Organon Teknika, Turnhout, Belgium) were used.

Differential Staining of Lymph and Blood Vessels

Four-micron cryosections were air dried and fixed in acetone at room temperature for 10 minutes. For the first staining reaction, they were incubated for 60 minutes at room temperature with undiluted PAL-E supernatant. After this and all following incubation steps, the sections were rinsed with ample phosphate-buffered saline (PBS). Then, the secondary 1:200 diluted biotinylated affinity-purified anti-mouse IgG (Vectastain, Vector Laboratories, Burlingame, CA) was incubated for 30 minutes, followed by a 45-minute incubation with alkaline phosphatase-biotin avidin complex (ABC mouse IgG kit, Vectastain). The first staining was developed for 10 minutes with a mixture of 1 mg/ml Fast Blue, 0.2 mg/ml naphthol phosphate, and 0.24 mg/ml levamisole (Sigma-Aldrich, Bornem, Belgium). For the second staining, the anti-CD31 antibody, diluted 1:2000, was incubated for 60 minutes, followed by a 30-minute incubation of 1:100 dilution of horseradish-peroxidase-conju-

gated rabbit anti-mouse immunoglobulin antibody (DAKO immunoglobulins, Copenhagen, Denmark). The second staining was developed by a 10-minute incubation with 0.4 mg/ml amino-9-ethyl-carbazole solution (Aldrich, Steinheim, Germany). In control sections, one or both of the primary antibodies were omitted. Nuclei were counter-stained for 2 minutes at room temperature with 1% methyl green (Zymed, San Francisco, CA), and the sections were mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

Determination of Lymph and Blood Vessel Density

The number of lymphatics and blood vessels was determined in sections of normal skin (n = 14) and in melanoma (n = 27) biopsies by visual examination at ×100 magnification by two independent observers. The combination of the delicate red PECAM-1 staining signal and the presence of melanin precluded the use of automated image analysis techniques. Vascular counts included complete cross sections, partial cross sections, and small groups of positive cells. In the melanoma sections, vascular densities could be determined in tumor-free fields in 10 cases that are indicated as uninvolved skin. Melanoma sections from areas outside the lesion (n = 3) were also included as uninvolved skin. In 10 cases, the biopsies contained sufficient areas of both uninvolved skin and tumor to allow for vascular density determination. Tumor vascular densities were determined in both HGPM (n = 12) and VGPM (n = 12). For each determination, at least three nonoverlapping fields were counted; the mean number of fields counted per section was 4.9 ± 2.1. For statistical analysis, the Prism and Instat software programs (GraphPad) were used.

Indirect Immunoelectron Microscopy

The samples for immunoelectron microscopy were processed as described previously.[16,18]

Results

Differential Staining of Lymph Vessels

To circumvent the lack of a specific marker for lymphatic endothelial cells, a protocol was designed based on the reactivity of anti-CD31 antibodies with both lymphatic and blood vessel endothelial cells in combination with the reactivity of a second antibody with blood vessel endothelium alone. Three blood vessel markers were tested. Staining with MAb PAL-E was superior to staining with anti-vWF/FVIIIrA pAb, anti-Coll-IV pAb, or antl-CD34 MAb. Anti-vWF and, to a lesser extent, anti-Coll-IV also reacted with lymphatic endothelium, and both the anti-vWF pAb, the anti-Coll-IV pAb, and the anti-CD34 MAb produced high background staining (not shown). Efforts to reduce background by dilution to improve specificity were unsuccessful. Therefore, all further experiments were carried out by using PAL-E

Staining Patterns in Skin Biopsies

Differential staining of normal skin showed a number of blood capillaries that were intensely PAL-E positive and a number of CD31-positive, PAL-E-negative ($CD31^+/PAL\text{-}E^-$) vascular structures that, on the basis of their architecture and characteristic staining pattern, were classified as lymphatic vessels (Figure 1a). This staining pattern is caused by high CD31 expression on inter-endothelial junctions, as has been described previously for blood vessel endothelium.[19] Recently, a similar pattern was shown to occur in lymphatic endothelium by staining marker expression in the context of ultrastructural morphology.[16] Immunoelectron microscopic examination of sections of melanoma biopsies labeled with anti-CD31 also showed the junctional staining pattern (Figure 2). Often, lymphatic staining was completely restricted to these junctions, yielding an extremely delicate pattern. Arteries and arterioles were not or only faintly stained by PAL-E, as described previously,[17] but could be identified by vessel wall morphology. In addition to the blood vessel endothelium, PAL-E stained the basal lamina of the epidermis.[20] Lymphatic vessels were ubiquitously present in high dermal positions, representing the subepidermal lymphatic plexus, or accompanying a blood capillary, with a lower overall density than blood vessels. Often, the lymphatic lumen was compressed, but also very large open lymphatic vessels were observed.

Staining Patterns in Biopsies of Human Cutaneous Melanoma Lesions

Differential double staining of sections from primary cutaneous melanoma lesions showed the tumor-associated blood and lymphatic vasculature. Extensive blood capillary formation (hemangiogenesis) was observed in cases of VGPM and occurred throughout the tumor mass. Often, the tumor was organized in cell nests surrounded by stromal septa containing the blood capillaries (Figure 1b). In all biopsies, lym-

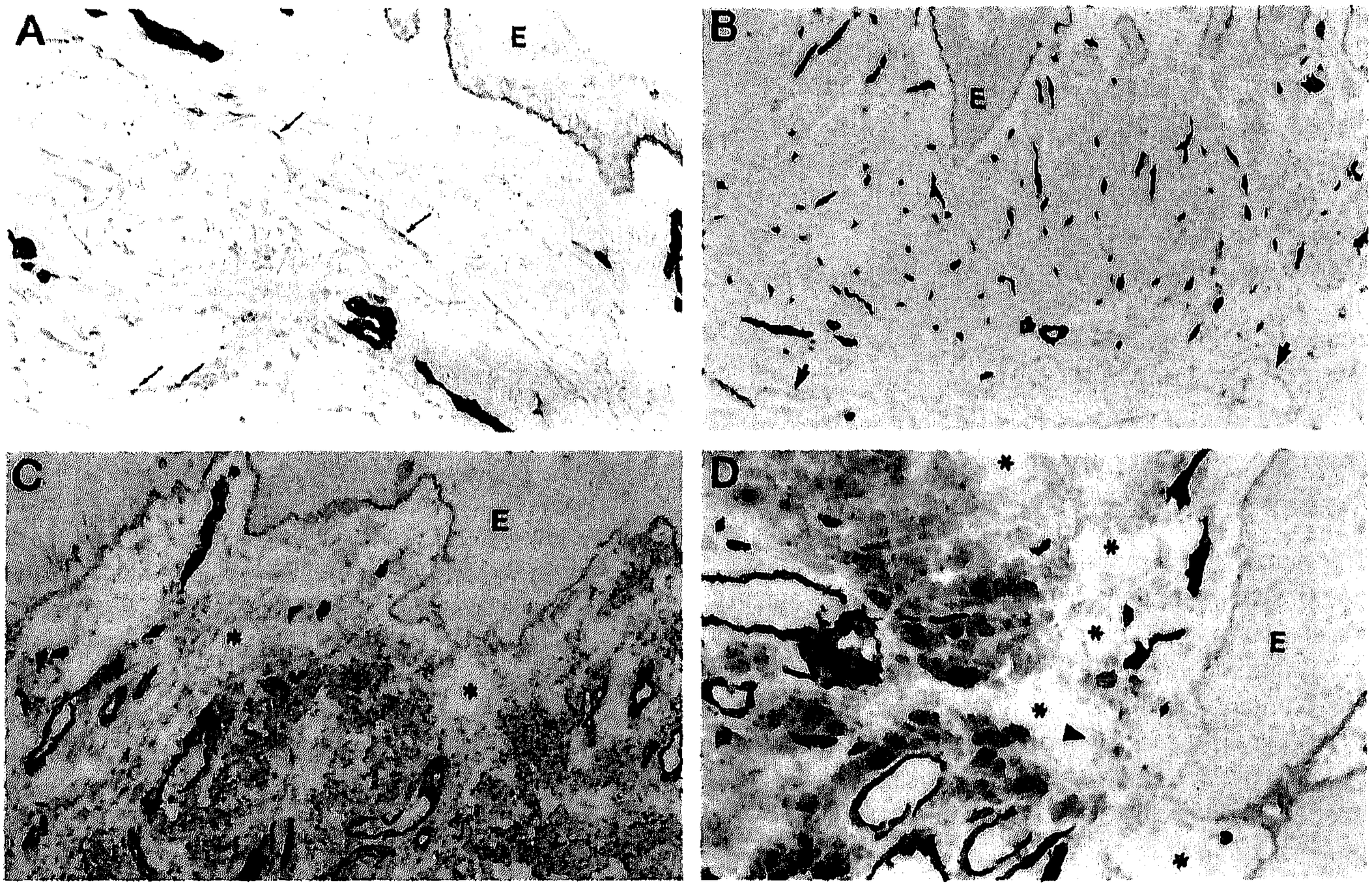

Figure 1. *Differential staining of blood and lymph vessels in frozen sections. Blood vessels are stained blue by MAb PAL-E, lymph vessels red by the anti-CD31 MAb.* a: *Normal skin.* Arrows *indicate junctional staining of lymphatic endothelial cells by the anti-CD31 MAb.* b: *Primary cutaneous melanoma, vertical growth phase. Note the nodular organization of the tumor and the presence of blood capillaries in stromal septa.* Arrows *mark lymphatic vessels present in the subtumor area.* c: *Primary cutaneous melanoma, vertical growth phase.* Asterisks *mark the lumina of intratumor lymphatics in a subepidermal position. In tumor areas, the PAL-E antigen is induced, resulting in a blue staining.* d: *Primary cutaneous melanoma, vertical growth phase. Lymphatic vessels in a subepidermal position are surrounded by melanin-containing tumor cells. The* arrowhead *marks a disruption of the lymphatic vessel wall.* Asterisks *indicate lymphatic lumina. E, epidermis. Methyl green* (a, b, *and* d) *and hematoxylin* (c) *counterstain; magnification, ×250.*

phatic vessels could be detected. Most of the lymphatic vessels were present in the subtumor zone and resembled normal pre-existing lymphatic vessels (Figure 1b). In addition, very delicate lymphatic clefts were present between tumor fields (Figure 1c) or at the site of the pre-existing subepidermal lymphatic plexus (Figure 1d), especially in cases where florid hemangiogenesis occurred. Often, the subepidermal lymphatics were completely surrounded by the expanding tumor mass and compressed by it. In Figure 3, the architecture of an expanding melanoma lesion is shown in a representative case. The directions of expansion and the area of lymphatic entrapment are indicated. A high-power micrograph of the latter area is shown in Figure 1c. Inside the tumor, signs of lymphatic invasion could be observed (Figure 1, c and d), although this was difficult to ascertain at the light microscopic level.

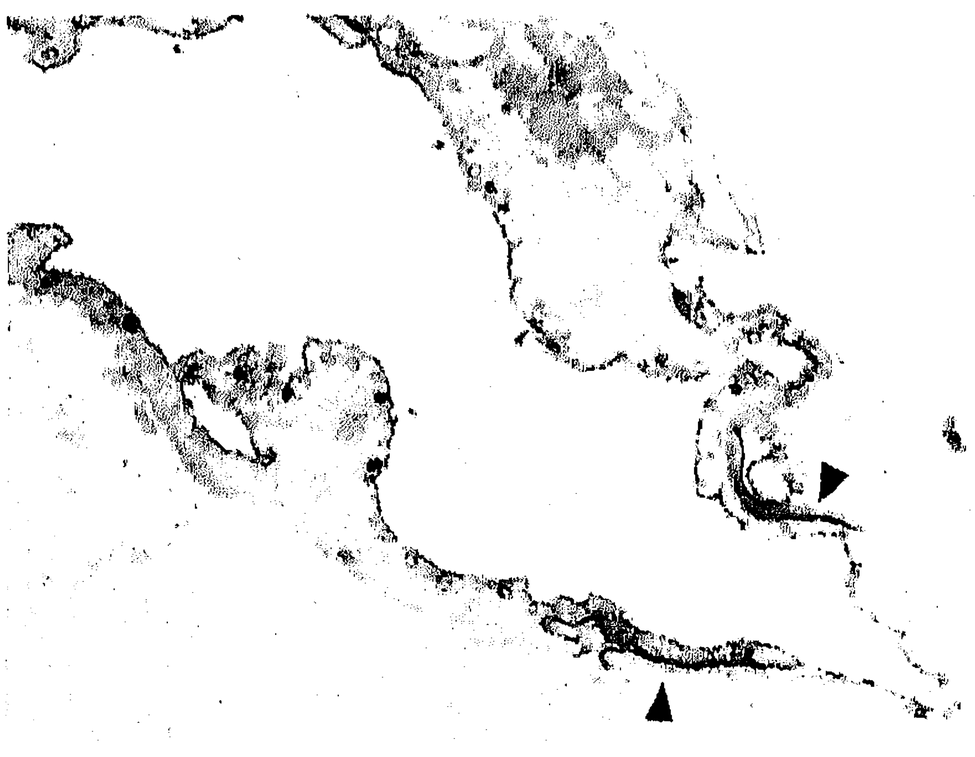

Figure 2. *Immunoelectron microscopy micrograph showing intercellular junctional staining of lymphatic endothelial cells of a lymph vessel in a melanoma biopsy by anti-CD31 MAb.* Arrowheads *indicate intercellular junctions. Magnification, ×20,000.*

Determination of Lymphatic and Blood Vessel Density

To quantify blood and lymphatic vessel density, vascular counting was performed in a number of differentially stained sections of normal skin, uninvolved skin from melanoma patients and from primary cutaneous melanoma. Figure 4 shows that the mean

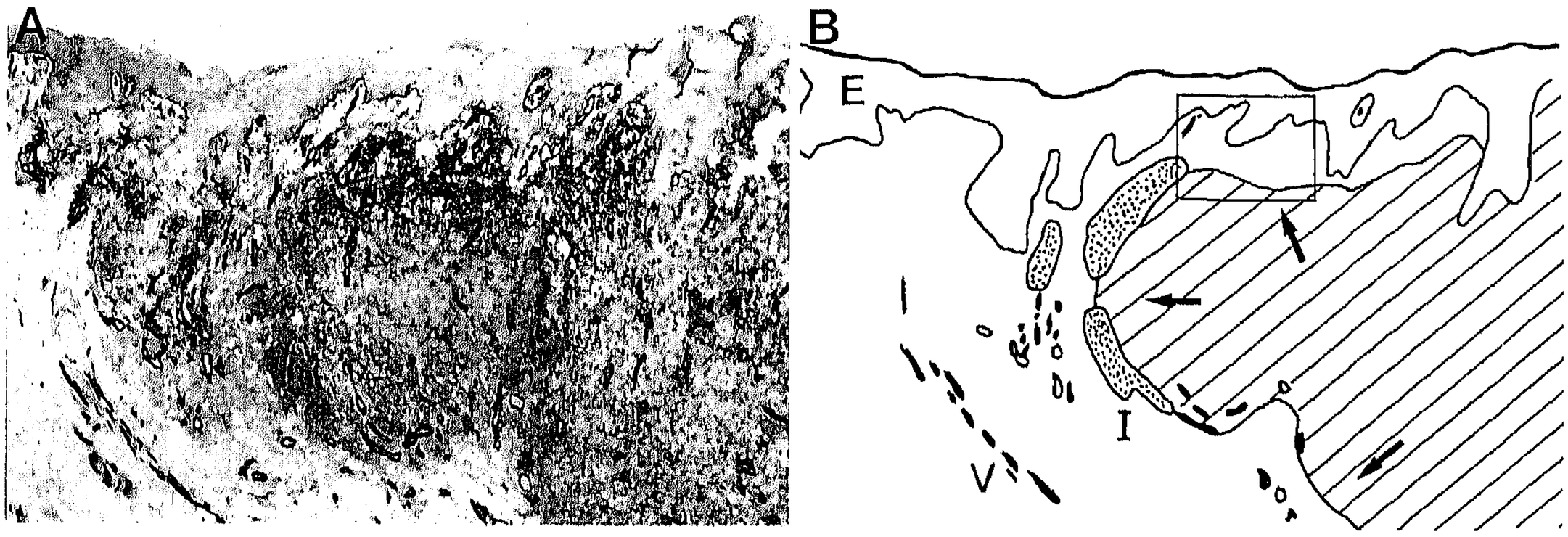

Figure 3. *Architecture of an expanding cutaneous melanoma lesion.* a: *Differential staining with PAL-E and anti-CD31.* b: *Schematic drawing of the expanding lesion shown in* a. *The* hatched area *represents the tumor mass.* Arrows *indicate the directions of expansion. The* boxed area *is shown in Figure 1c; in this area, lymph vessels are trapped. E, epidermis; I, mononuclear infiltrate (dotted); V, blood vessels. Magnification, ×100.*

blood capillary density was already significantly increased ($P < 0.01$) in HGPM compared with uninvolved skin. Very high blood capillary densities ($P < 0.001$) were observed in VGPM, indicating a local process of active hemangiogenesis in these tumor lesions. Mean lymphatic density in normal and uninvolved skin was considerably lower than the mean blood vessel density (15 *versus* 80 cross sections per field). Lymphatic density in both HGPM and VGPM was comparable to that in normal and uninvolved skin ($P > 0.05$). Counting of the lymphatic and blood capillary density in the same section enabled us to calculate the ratio between these densities for each individual melanoma specimen. Comparison of these ratios showed a linear correlation that was comparable between normal skin, uninvolved skin, and HGPM (Figure 5). In VGPM, this correlation was lost, due to the high and variable levels of hemangiogenesis.

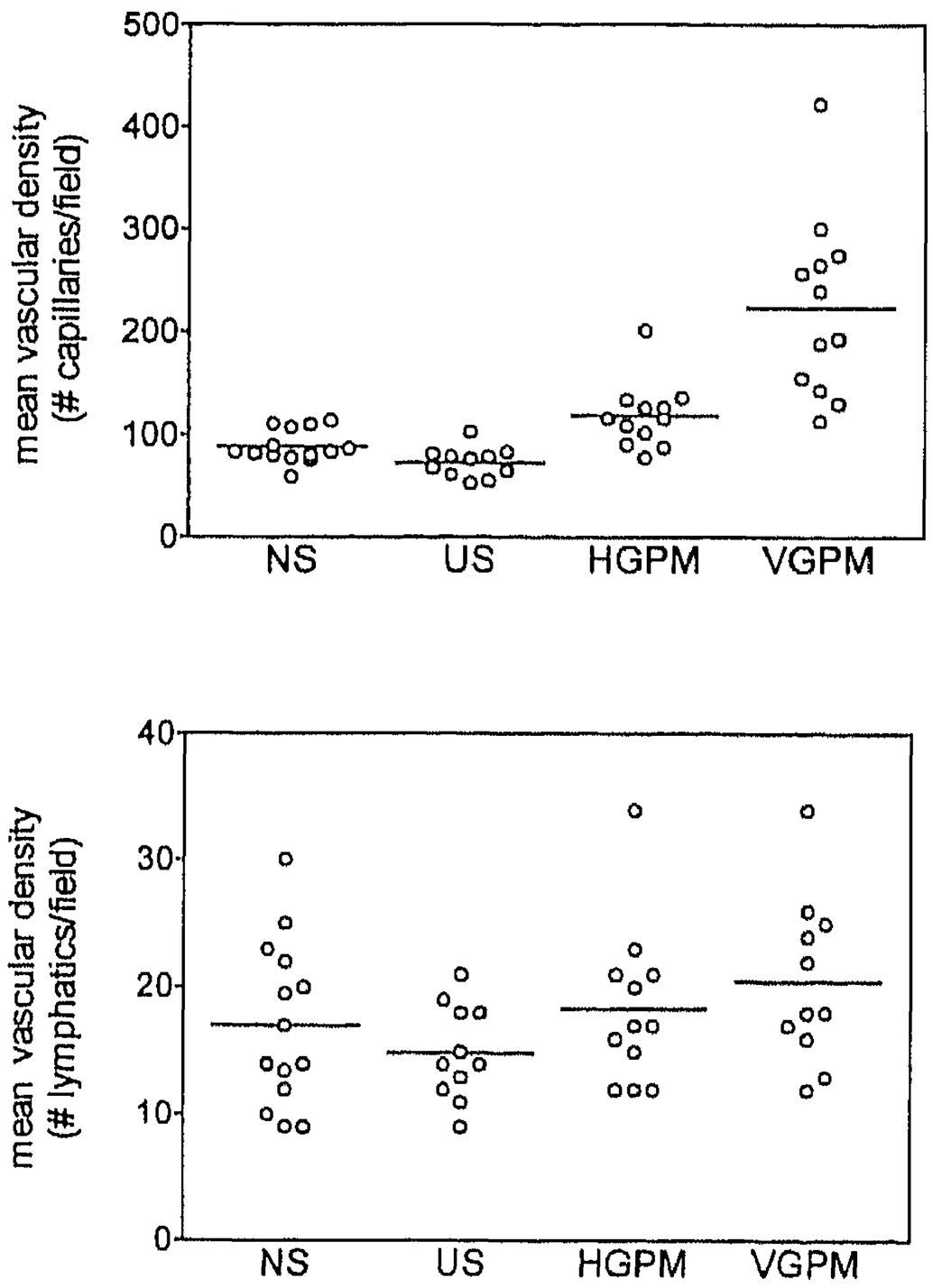

Figure 4. *Blood* (top) *and lymph* (bottom) *vessel density in normal skin (NS), uninvolved skin (US), horizontal growth phase primary cutaneous melanoma (HGPM), and vertical growth phase primary cutaneous melanoma (VGPM). Each point represents the mean vascular density in a single biopsy. The lines represent the overall mean vascular density in the indicated category. Levels of significance of the difference of blood vessel density compared with that measured in normal skin (Kruskal-Wallis nonparametric analysis of variance and Dunn's multiple comparisons test) were as follows: uninvolved skin, not significant; HGPM, not significant; VGPM, $P < 0.001$. Levels of significance compared with uninvolved skin were as follows: HGPM, $P < 0.01$; VGPM, $P < 0.001$.*

Discussion

To study the lymphatic vasculature in cutaneous lymphoma, we developed a staining protocol that differentially highlights the blood and lymph vasculature in frozen sections. In our hands (results not shown), this method was superior to a previously described protocol based on differential expression of *Ulex europaeus* agglutinin I and collagen type IV.[13]

The question arises whether the $CD31^{+}/PAL\text{-}E^{-}$ vascular structures are indeed lymph vessels and, if so, whether all lymph vessels are identified by this staining. The selectivity of the staining strongly depends on the specificity of the PAL-E MAb. In a recent immunoelectron microscopic investigation, we were able to identify lymphatic vessels by morphological criteria and study the expression of a variety of vascular markers.[16] No staining of these vessels with the marker PAL-E was observed in any case, but they were invariably stained with anti-CD31 antibody, showing the characteristic intense staining

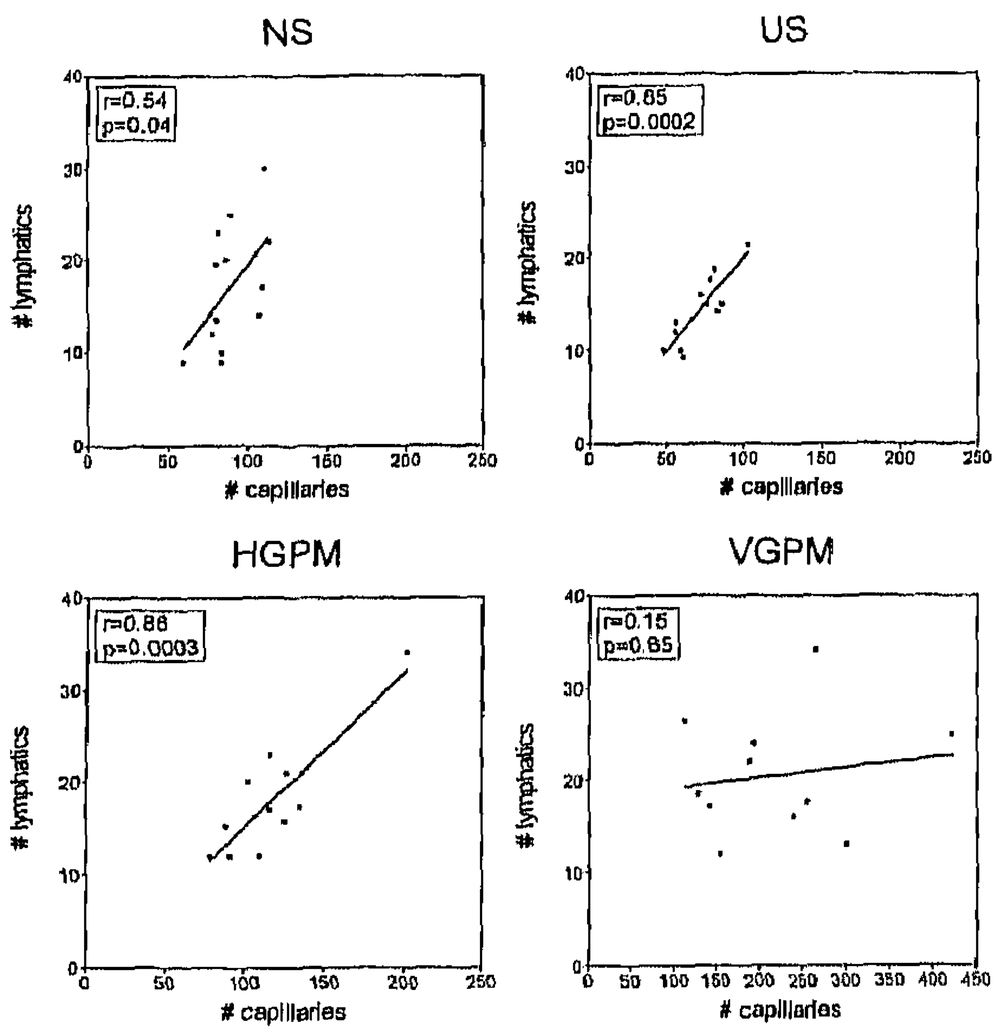

Figure 5. *Ratios between blood and lymph vessel density of normal skin (NS), uninvolved skin (US), and horizontal (HGPM) and vertical (VGPM) growth phase primary cutaneous melanoma. Each dot represents the ratio between the mean vascular densities in a single biopsy. In each panel, the best fitting straight line is drawn and the corresponding values for correlation (r) and level of significance (p) are indicated.*

of intercellular junctions. On the basis of this study, we concluded that the $CD31^{+}$/PAL-E^{-} vascular structures were lymph vessels and that all lymph vessels are decorated by the staining. In a few cases, a mononuclear infiltrate was stained by the anti-CD31 MAb, which may have interfered with the identification of lymphatics in these areas.

The reason for the absence of lymphangiogenesis in the presence of extensive hemangiogenesis is unclear. Often, the tumor mass was completely devoid of lymphatic vessels, which then only were found in peritumor dermal regions. It may be speculated that receptors for angiogenic factors are lacking on lymphatic endothelium or that the lack of perivascular cells in the lymph vasculature is of crucial importance, but both these options need further study. It is hard to imagine that the extremely high blood vessel densities that we observed in several cases of VGPM are not related to metastatic incidence. However, blood vessels were often surrounded by perivascular cells and extracellular matrix, and the melanoma cells were frequently organized in tumor nodules separated by a second basal lamina from stromal components. This does not explain a preference for lymphatic spread completely, but the morphological findings suggested that lymphatic invasion occurred when part of the relatively dense subepidermal lymphatic plexus was trapped between the expanding tumor mass and the epidermis and was invaded by the ascending melanoma front. Lymphatic vessels in the subtumor region were not trapped but were pushed away by the expanding tumor mass. This model for lymphogenic spread provides an explanation for the paradox that melanoma progression requires hemangiogenesis but that prognosis is not related to the blood capillary density.[6] It also suggests that lymphatic invasion occurs in a particular area, the subepidermal plexus. The local architecture of the tissue around the tumor and the intrinsic density of the lymphatic bed thus may influence prognosis considerably and in this way obscure a possible relation with blood capillary density. In addition, as lymphatic invasion occurs at the interphase of tumor and normal tissue, the relation between hemangiogenesis and prognosis may be lost in the more advanced melanoma lesions where the volume of the interphase has become relatively small compared with the tumor mass. Recently, blood vessel density was described as a prognostic parameter in stage I cutaneous melanoma lesions[11] in contrast to earlier work where such a relation was not found in a larger series including more advanced lesions.[6]

In other tumor types such as breast carcinoma a similar scenario as in melanoma could be operative as well. Although a relation between capillary density and prognosis was established in this tumor type, it cannot be ignored that lymph node metastasis is a frequent complication and an important independent negative parameter of prognosis. We hypothesize that here the relation between capillary density and lymphatic invasion is a directly proportional one because it is not influenced by architectural conditions such as the presence of a unidirectional barrier like the epidermal layer that limits expansion in all dimensions. Further investigation of breast carcinoma and other tumor types that preferentially spread initially via the lymphatic route is required to confirm this proposed model.

Acknowledgment

We thank F. Rietveld for technical support.

References

1. Folkman J: What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst 1990, 82:4–6
2. Liotta LA, Steeg PS, Stetler-Stevenson WG: Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 1991, 64:327–336

3. Weidner N, Semple JP, Welch WR, Folkman J: Tumor angiogenesis and metastasis: correlation in invasive breast carcinoma. N Engl J Med 1991, 324:1–8
4. Weidner N: Intratumor microvessel density as a prognostic factor in cancer. Am J Pathol 1995, 147:9–19
5. Carnochan P, Briggs JC, Westbury G, Davies AJ: The vascularity of cutaneous melanoma: a quantitative histologic study of lesions 0.85–1.25 mm in thickness. Br J Cancer 1991, 64:102–107
6. Busam KJ, Berwick M, Blessing K, Fandrey K, Kang S, Karaoli T, Fine J, Cochran AJ, White WL, Rivers J, Elder DE, Wen DP, Heyman BH, Barnhill RL: Tumor vascularity is not a prognostic factor for malignant melanoma of the skin. Am J Pathol 1995, 147:1049–1056
7. Leedy DA, Trune DR, Kronz JD, Weidner N, Cohen JI: Tumor angiogenesis, the p53 antigen, and cervical metastasis in squamous carcinoma. Otolaryngol Head Neck Surg 1994, 111:417–422
8. Srivastava A, Laidler P, Davies RP, Horgan K, Hughes LE: The prognostic significance of tumor vascularity in intermediate-thickness (0.76–4.0 mm thick) skin melanoma: a quantitative histologic study. Am J Pathol 1988, 133:419–423
9. Barnhill RL, Fandrey K, Levy MA, Mihm MC Jr, Hyman B: Angiogenesis and tumor progression of melanoma: quantification of vascularity in melanocytic nevi and cutaneous malignant melanoma. Lab Invest 1992, 67: 331–337
10. Graham CH, Rivers J, Kerbel RS, Stankiewicz KS, White WL: Extent of vascularization as a prognostic indicator in thin (≤0.76 mm) malignant melanomas. Am J Pathol 1994, 145:510–514
11. Rongioletti F, Miracco C, Gambini C, Pastorino A, Tosi P, Rebora A: Tumor vascularity as a prognostic indicator in intermediate-thickness (0.76–4 mm) cutaneous melanoma. Am J Dermatopathol 1996, 18:474–477
12. Deutsch A, Lubach D, Nissen S, Neukam D: Ultrastructural studies on the invasion of melanomas in initial lymphatics of human skin. J Invest Dermatol 1992, 98:64–67
13. Fallowfield ME, Cook MG: Lymphatics in primary cutaneous melanoma. Am J Surg Pathol 1990, 14:370–374
14. Folkman J: Angiogenesis and tumor growth (correspondence). N Engl J Med 1996, 334:920–921
15. Newman PJ, Berndt MC, Gorski J, White GC II, Lyman S, Paddock C, Muller WA: PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. Science 1990, 247:1219–1222
16. Erhard H, Rietveld FJR, Bröcker EB, de Waal RMW, Ruiter DJ: Phenotype of normal cutaneous microvasculature. J Invest Dermatol 1996, 106:135–140
17. Schlingemann RO, Dingjan GM, Emeis JJ, Blok J, Warnaar SO, Ruiter DJ: Monoclonal antibody PAL-E specific for endothelium. Lab Invest 1985, 52:71–76
18. McLean IW, Nakane PK: Periodate-lysine-paraformaldehyde fixative: a new fixation for immunoelectron microscopy. J Histochem Cytochem 1974, 22:1077–1081
19. Muller WA, Ratti CM, McDonnell SL, Cohn ZA: A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. J Exp Med 1989, 170:399–414
20. Ruiter DJ, Schlingemann RO, Rietveld FJR, de Waal RMW: Monoclonal antibody defined human endothelial antigens as vascular markers. J Invest Dermatol 1989, 93:25S–32S